

ajava

Asian Journal of Animal and Veterinary Advances



Academic
Journals Inc.

www.academicjournals.com

Blood Cell Histology of Horseshoe Crab, *Tachypleus gigas*

¹Sh. Shakibazadeh, ¹A. Christianus and ²P. Hajeb

¹Department of Aquaculture, Faculty of Agriculture, Universiti Putra Malaysia, 43400 Serdang, Selangor Darul Ehsan, Malaysia

²Centre of Excellence for Food Safety Research, Faculty of Food Science and Technology, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia

Corresponding Author: Annie Christianus, Department of Aquaculture, Faculty of Agriculture, Universiti Putra Malaysia, 43400 Serdang, Selangor Darul Ehsan, Malaysia Tel: 03-89474884 Fax: 03-89408311

ABSTRACT

Amoebocytes are the main carrier of innate immune system in horseshoe crabs. Cytoplasm of these cells are packed with granules which consist of all coagulation factors and anti-bacterial compounds. Light microscopy (using May-Grunwald and Giemsa staining technique) of 46 blood specimen for both sexes of *Tachypleus gigas* and observation under Transmission Electron Microscopy (TEM) were performed. Light microscopy revealed that these are ovoid cells containing blue nucleus surrounded by a cytoplasm packed with pink granules. Large and small axes measured 18.8 ± 0.5 and 12.8 ± 0.4 μm , respectively. Total blood cell count ($33000 \text{ cell mm}^{-3}$) does not show any significant difference between sexes. TEM of normal and degranulated blood cells revealed that *T. gigas* hemocytes consist of two type of granules (large and small). Blood cells have all the necessary organelles of normal cells. During the granule formation, fusion of different intermediate granules were observed. Degranulated hemocytes under light microscope and TEM showed cells containing large nucleus and cytoplasmic granules were replaced with a large cytoplasmic vacuole. In light and electron microscopy only one type of hemocyte was observed, the granular cells. These granular cells consist of large and small granules.

Key words: *Tachypleus gigas*, horseshoe crab, granular blood cell, histology, amoebocytes

INTRODUCTION

Invertebrates are thought to rely solely on their innate immune system (Kurtz, 2005). The circulating hemolymph in marine invertebrate contains biologically active substances such as lectins, anti-coagulant factors and anti-bacterial compounds. All of these factors contribute to the innate immune system in marine invertebrates against invading microorganisms which can reach to 10^8 bacteria mL^{-1} and 10^9 virus mL^{-1} in the sea water. The survival of marine invertebrates in this environment suggests a highly effective innate immune system (Tincu and Taylor, 2004).

In the horseshoe crab this innate immune system carried mainly in the hemolymph (Miyata *et al.*, 1989) contained hemocytes or amoebocytes. Cytoplasm of these cells are packed with granules including all the factors required for blood coagulation which comprised of clottable protein and antibacterial compounds (Murer *et al.*, 1975).

The blood of *Limulus polyphemus* contains only one type of cell, amoebocyte (Conrad *et al.*, 2004). Under the transmission and scanning electron microscopy, the cytoplasm of the

Limulus polyphemus amoebocytes are seen packed with only one type of granule (Murer *et al.*, 1975). In *Tachypleus tridentatus* there are two types of hemocytes, non-granular and granular hemocytes. The latter contained numerous dense granules which are classed into two major types, large and small granules. The large granules contain three clotting factors and one anti-microbial compound and the small granules exclusively contained the other anti-microbial compounds (Toh *et al.*, 1991). The intermediate granules were reported by Copeland and Levin (1985) in *L. polyphemus*, suggested that these are the intermediate stages for large granules. While Toh *et al.* (1991) described four types of Golgi vacuoles in *Tachypleus tridentatus* with various sizes and contents.

There are only four species of extant horseshoe crab. One; *Limulus polyphemus* in North and Central America (Botton and Rops, 1987), and the other three; *Tachypleus tridentatus*, *Tachypleus gigas* and *Carcinoscorpius rotundicauda* in South East Asia (Chiu and Morton, 1999).

In this study the blood cells of *Tachypleus gigas*, one of the species found in Peninsular Malaysia, were investigated through Light microscope and Transmission electron microscopy (TEM).

MATERIALS AND METHODS

In blood cell count and smear preparation, a total of 46 horseshoe crab specimens (22 females and 24 males) used were bought from fishermen in Pulau Lumut, Selangor, Malaysia during November 2004 to April 2005.

The blood was extracted by cardiac puncture. For blood extraction animals were fixed onto a rack and bent with dorsal surface towards front. The muscles above the heart were exposed and swabbed with 70% ethanol. Disposable pyrogen free 1 mL syringes and 25G needles were used to draw 0.2 mL hemolymph and then diluted to 1 mL with pre-chilled 10% formalin (in autoclaved seawater). The diluted hemolymph was stirred evenly by inversion and kept chilled for 1-2 h. The diluted hemolymph was used for blood cell count as well as smear preparation.

Blood cell count (repeated three times) of the diluted specimen was carried out using hemocytometer.

Blood smear was prepared by dropping the diluted blood onto 70% ethanol pre-cleaned slides and a clean slide used at 30-40° angle to prepare blood smear (Miy and Sparks, 1980). Each specimen has three replications. The slides were fixed for 1-2 min by absolute methanol, followed by May-Grunwald stain for 5-7 min, followed by Giemsa for 20-30 min.

Blood cell measurement was carried out using a total of seven specimens (each containing three replications). Both axes of the oval shaped granulocyte of 50 cells per slides were measured randomly. The measurements of red blood cells and photography were carried out using Image analyzer, PC_image (vga 24) software, Nikon microscope (Eclipse E 200) and a Nikon camera (Cool Pix 995).

TEM of fresh extracted blood was investigated (under extremely sterilized condition) for normal blood cell before and after degranulation. For normal blood cell before degranulation, a 0.5 mL of freshly extracted blood was immediately added into 10 mL volume of 3% glutaraldehyde in 0.1 M cacodylate buffer solution containing 0.1 M glucose for osmoregulation at 4°C. The cells were pre-fixed for 2 h then centrifuged at 500 rpm for 30 min, washed three times with 0.1 M cacodylate buffer (with 10 min interval). The cells then post-fixed in 1% osmium tetroxide (OsO₄) in the same buffer solution for 3 h at 4°C. Same procedure carried out for clotted blood to view the cells after degranulation.

RESULTS

Under light microscope, blood cells of *T. gigas* were ovoid with light pink cytoplasm packed with red granules. A circular or ovoid blue nucleus was observed in the cytoplasm (Fig. 1). After the discharged of granules, the cytoplasm was empty. Some pseudopodia were observed to form and extended outward. A round and blue nucleus was observed (Fig. 2). Non-granular blood cells were extremely rare in *T. gigas* hemolymph. This cell consisted of a large purple nucleus which occupied most of the cytoplasm space. A thin crescent strip of light blue or purple cytoplasm was observed. There was no granule distinguished in the cytoplasm of this cell (Fig. 3).

The blood cell count ranged from 10,000-60,000 cell mm^{-3} , with an average of 33,000 cell mm^{-3} (29,000 for females and 37,000 for males). Blood cell count of the sexes was compared using student t-test. There was no significant difference ($p>0.05$) between the blood cell counts of the sexes of *T. gigas*.

Blood cell measurements of *T. gigas* in terms of large and small axes were 18.8 ± 0.5 and 12.8 ± 0.4 μm , respectively.

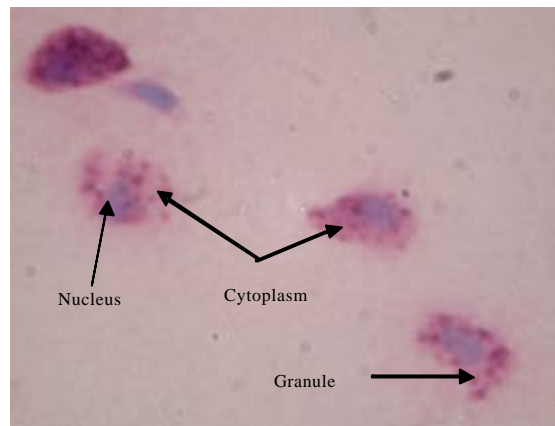


Fig. 1: Light-microscopic image of normal granular blood cell of *Tachypleus gigas* magnified at 1000x

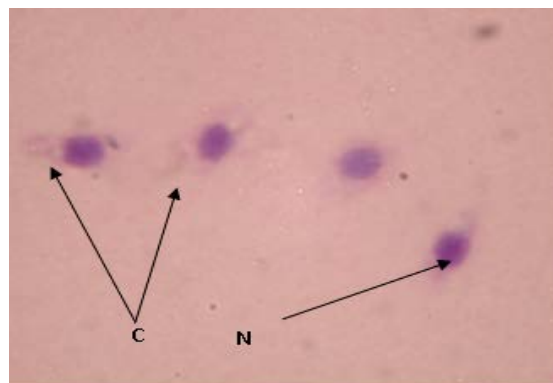


Fig. 2: *Tachypleus gigas* granular blood cells after the discharge of granules, magnified at 1000x, N: Nucleus, C: Cytoplasm

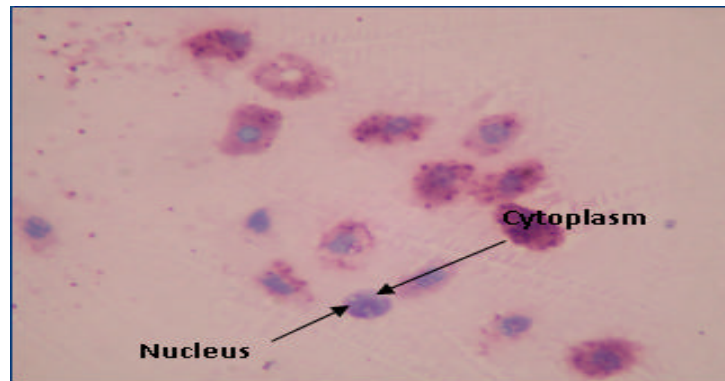


Fig. 3: Nongranular blood cell of *Tachypleus gigas* magnified at 1000x

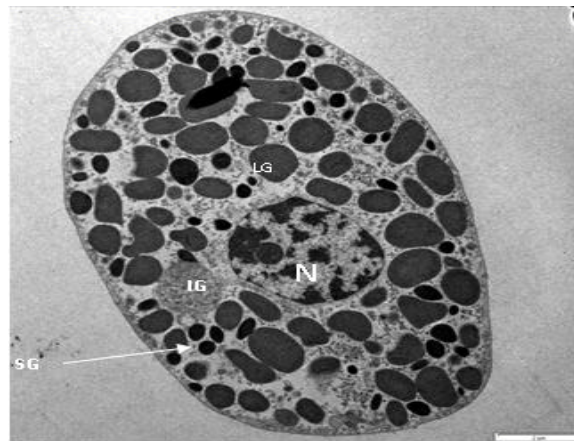


Fig. 4: Normal granular blood cell of *Tachypleus gigas*, under TEM magnified at 1000x, N: Nucleus, IG: Intermediate granule, LG: Large granule, SG: Small granule

TEM of normal granular cells of *T. gigas* revealed that the cytoplasm consisted of large (1 μm) and small (0.5 μm) granules. A large nucleus observed in the cytoplasm (Fig. 4). A well developed Golgi apparatus appeared close to the nucleus. The electron density of Golgi cisternae increased from the *cis* to the *trans* side with many small Golgi vesicles presented around the cisternae (Fig. 5b-e). Several spherical or oval shaped mitochondria were observed in the cytoplasm (Fig. 5f-h). Different intermediate granules as described by Toh *et al.* (1991) were tubular (T-granule), condense electron-dense (C-granule) and granules which contained patches of electron-dense material scattered in the lumen (P-granules). Moreover an intermediate granule with amorphous pattern of content was observed (Fig. 5a and b). Fusion of different intermediate granules such as, fusion of C and P-granule, C and T-granule (Fig. 5b, d and e) and fusion of two C-granules (Fig. 5 d) were observed during the granules maturation.

Transmission Electron Microscopy of the granular blood cells after the discharged of granules is showed by the empty cytoplasm (no more granules remained in the cytoplasm).

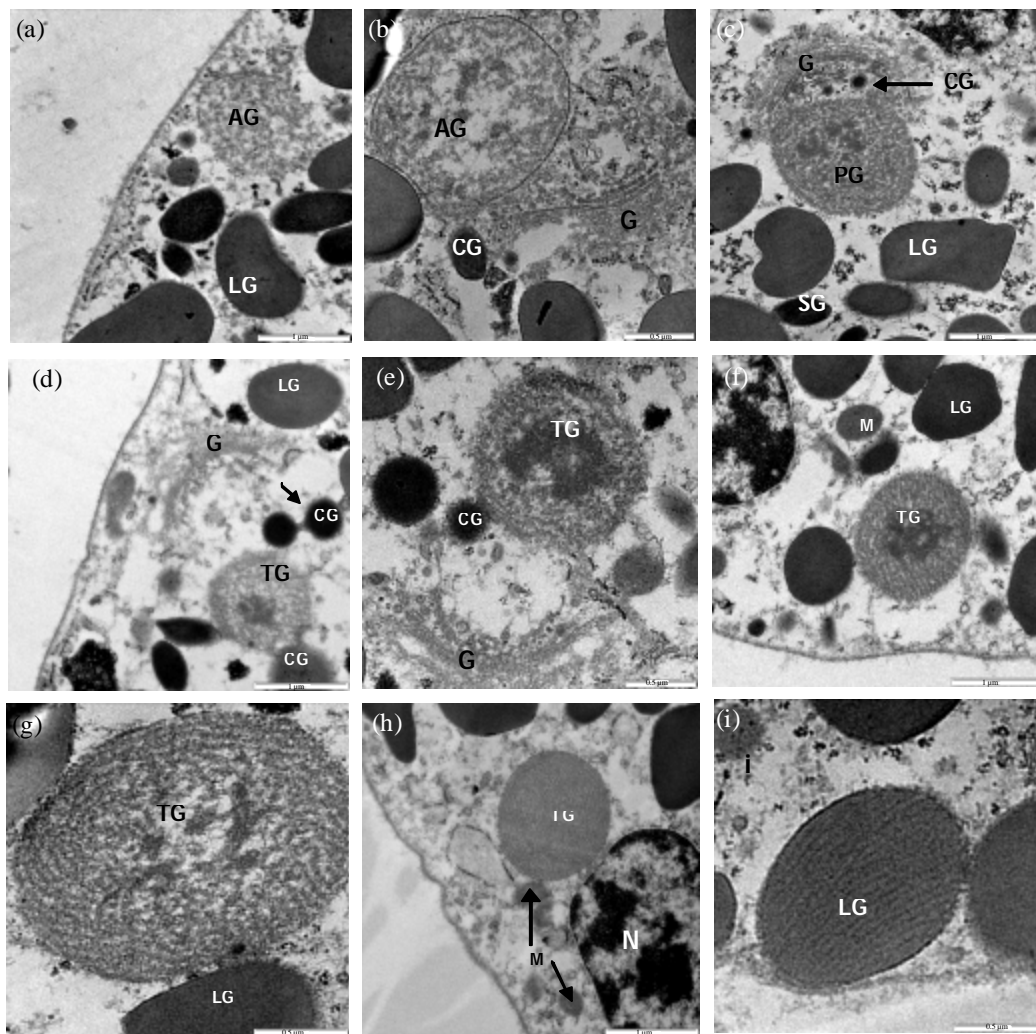


Fig. 5(a-i): Transmission of electron microscopy of *Tachypleus gigas* of granular blood cell, (a) An amorphous intermediate granule (AG) and large granule (LG), 4000x, (b) An amorphous intermediate granule (AG) containing few electron dense particles evaginated toward C-granules (CG) and Golgi apparatus (G), 6300x, (c) An Intermediate P-granule containing more regular pattern and dense particles (PG), Golgi apparatus (G), C-granule (CG), large (LG) And small granule (SG), 4000x, (d) Fusion of an intermediate T-granule containing an electron dense core (TG) with a C-granule (CG), Golgi apparatus (G), large granule (LG) and fusion of two C-granules (CG and arrow), 4000x, (e) Fusion of an intermediate T-granule containing an electron dense core (TG) in tubular pattern with a C-granule (CG) and Golgi apparatus (G), 4000x, (f) An intermediate T-granule containing an electron dense core (TG), mitochondria (M), Large granule (LG), 4000x, (g) An intermediate T-granule containing electron dense particle in tubular pattern (TG), Large granule (LG), 8000x, (h) An intermediate T-granule containing very regular and dense tubular pattern (TG), Mitochondria (M) and nucleus (N), 4000x, (i) A large granule (LG) containing tubular pattern, 8000x

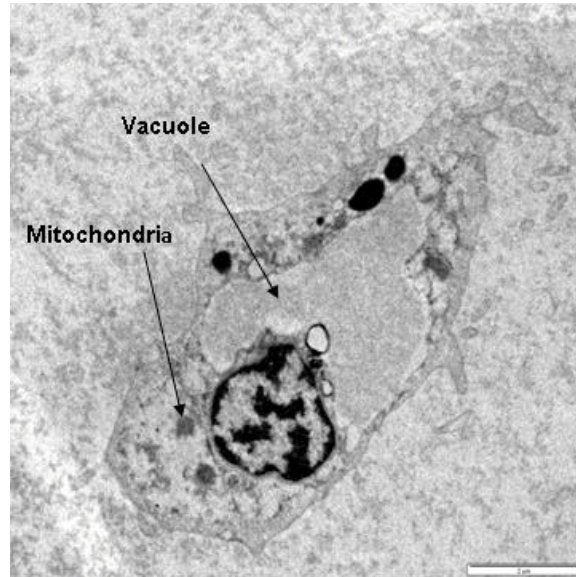


Fig. 6: Transmission electron microscopy of *Tachypleus gigas* granular cell after the discharged of granules, magnified at 1300x

Numerous vesicles was observed around a large and prominent nucleus. A large vacuole appeared in the cytoplasm of the granular cells after the discharged of their granules (Fig. 6).

DISCUSSION

Almost all the blood cells circulating in the hemolymph of the *T. gigas* are granulocytes. Non-granular cells. These cells are extremely rare (only one cell was observed during this study). Since, the respiratory pigment, hemocyanin is located extracellularly (Synder and Mangum, 1982) therefore, no mechanisms to transport the respiratory pigment like those of red blood cells in higher animals.

The average blood cells count of *T. gigas* was $33,000 \text{ cell mm}^{-3}$ (29,000 for females and 37,000 for males). It was almost similar range with blood cell count of *L. polyphemus* (20,000-60,000 cell mm^{-3}) as reported by Armstrong (1985). There was no significant difference between blood cell counts of sexes of the *T. gigas* using Student t-test.

The granulocytes were mostly oval or round, consisting of a large nucleus. The average granular cells size in terms of large and small axes were $18.8 \mu\text{m} \pm 0.5$ and $12.8 \mu\text{m} \pm 0.4$, respectively. The large axes measurements of *T. gigas* were within similar range (8-25 μm) to that reported by Sekiguchi (1988) and Conrad *et al.* (2004).

TEM observation revealed that the normal granular blood cells of horseshoe crabs, *T. gigas*, contained all the organelles of a well equipped cell which includes a nucleus, mitochondria, Golgi apparatus, endoplasmic reticulum, ribosome particles and microtubules. These observations were similar to those studies on *L. polyphemus* carried out by Dumont *et al.* (1966), Copeland and Levin (1985), Conrad *et al.* (2004) and on *T. tridentatus* by Jakobsen and Suhr-Jessen (1990) and Toh *et al.* (1991).

The granular cells were large and ovoid to fusiform, containing a large oval-shaped nucleus. The cytoplasm was packed with two types of granules, large and small. The average sizes for large

and small granule were 1 and 0.5 μm in diameter, respectively. These measurements were similar to those reported for *T. tridentatus*, up to 1.5 μm and less than 0.6 μm for large and small granule, respectively (Toh *et al.*, 1991) and *L. polyphemus*, 1-2 μm (Conrad *et al.*, 2004). Presence of two types of granules in the granulocytes of *T. tridentatus* was reported by Toh *et al.* (1991) and Shigenaga *et al.* (1993) while Copeland and Levin (1985) reported that the small granules were not observed in all of the amoebocytes in *L. polyphemus*.

Normal granular blood cell observation in *T. gigas* showed a large well developed Golgi apparatus and its associated structure. A large recently made intermediate granule was usually observed closed to the Golgi complex. These intermediate granules mostly showed tubular (T-granule) and/or amorphous pattern in different densities which consist of electron dense core. The amorphous granules probably are early stage for P-granule, since, fusion with electron dense granule (C-granule) resulted in increased of electron density of the content and electron dense core inside the granule will appeared. Fusion of dense granules (C-granule) to the intermediate amorphous (P-granule) and tubular granules (T-granule) and even fusion of two electron dense granules (C-granule) were observed.

Copeland and Levin (1985) and Toh *et al.* (1991) suggested that the tubular granules are the intermediate form for large granules in *L. polyphemus* and *T. tridentatus*. Fusion of P and C-granule in *T. tridentatus* leads to the theory of small granule formation by Toh *et al.* (1991) however, this theory was not suggested in *L. polyphemus*.

Mitochondria appeared oval or spherical and possessed typical cristae and a relatively dense matrix which is similar to the observation by Dumont *et al.* (1966).

Rough endoplasmic reticulum appeared in different areas of the cytoplasm, mostly close to the nucleus and Golgi apparatus.

Although, studies on decapods crustacean and scorpion hemocytes in 1990 by Hose *et al.* (1990) and Ravindranath (1974), respectively, showed that the hemolymph consisted of two types of cells in crustacean and seven classes of cells in scorpion. TEM observation of normal blood cells of *T. gigas* did not show non-granular blood cells and it was rarely seen under light microscope.

TEM observation of clotted blood cells revealed that almost all two populations of granules were discharged. Cytoplasmic area was filled by different size vacuoles, some cytoplasm evaginations appeared, mitochondria and other organelles were seen in the medullary area of the cell, similar to those reported by Dumont *et al.* (1966).

Although, the granulocytes did not exhibit morphological differences (Sekiguchi, 1988), some usually discharge all their granules in respond to un-sterile condition while some remain intact. It is most likely that the activity of these granulocytes is due to their different physiological roles in the hemolymph.

CONCLUSION

Granular blood cell is the only cell found in the hemolymph of *T. gigas*. This cell consists of two types of granules, large and small. Amorphous intermediate granule seems to develop their structures during fusion with electron dense granules to tubular granule which is known as an intermediate granule for large granule. Small granule formation might be due to fusion of electron dense granules.

ACKNOWLEDGMENT

Authors thank the government of Malaysia for funding this project through the Intensified Research on Priority Areas (IRPA) grant No. 01-02-04-0864 EA001.

REFERENCES

- Armstrong, P.B., 1985. Adhesion and Motility of the Blood Cells of *Limulus*. In: Blood Cells of Marine Invertebrates: Experimental Systems in Cell Biology and Comparative Physiology, Cohen, W.D. (Ed.). A.R. Liss Inc., USA., ISBN: 9780845122051, pp: 77-124.
- Botton, M.L. and J.W. Rops, 1987. Population of horseshoe crabs, *Limulus polyphemus*, on the Northwestern Atlantic continental shelf. Fish Bull., 85: 805-812.
- Chiu, H.M. and B. Morton, 1999. The distribution of horseshoe crabs (*Thachypleus tridentatus* and *Carcinoscorpius rotundicauda*) in Hong Kong. Asian Mar. Biol., 16: 158-169.
- Conrad, M., J. Denobile, I. Cheikhoutdinov, D. Escribano, K. Lee and W.D. Cohen, 2004. Cytoskeletal organization of *Limulus* amoebocytes pre- and post-activation: Comparative aspects. Biol. Bull., 207: 56-66.
- Copeland, D.E. and J. Levin, 1985. The fine structure of the amoebocyte in the blood of *Limulus polyphemus*, morphology of the normal cell. Biol. Bull., 169: 449-457.
- Dumont, J.N., E. Anderson and G. Winner, 1966. Some cytologic characteristics of the hemocytes of *Limulus* during clotting. J. Morphol., 119: 181-207.
- Hose, J.E., G.G. Martin and A.S. Gerald, 1990. A decapod hemocyte classification scheme integrating morphology, cytochemistry and function. Biol. Bull., 17: 33-45.
- Jakobsen, P.P. and P. Suhr-Jessen, 1990. The horseshoe crab *Tachypleus tridentatus* has two kinds of hemocytes: Granulocytes and plasmatocytes. Biol. Bull., 178: 55-64.
- Kurtz, J., 2005. Specific memory within innate immune systems. Trends Immunol., 26: 186-192.
- Miy, M.C. and A.K. Sparks, 1980. Hemocyte classification and differential counts in the Dungeness crab, *Cancer magister*. J. Invertebr. Pathol., 33: 134-143.
- Miyata, T., F. Tokunaga, T. Yoneya, K. Yoshikawa and S. Iwanaga *et al.*, 1989. Antimicrobial peptides, isolated from horseshoe crab hemocytes, tachyplesin II and polyphemusins I and II: Chemical structures and biological activity. J. Biochem., 106: 663-668.
- Murer, E.H., J. Levin and R. Holm, 1975. Isolation and studies of the granules of the amoebocytes of *Limulus polyphemus*, the horseshoe crab. J. Cell Physiol., 86: 533-542.
- Ravindranath, M.H., 1974. The hemocyte of a scorpion, *palamnaeus swammerdami*. J. Morphol., 144: 1-10.
- Sekiguchi, K., 1988. Hemocytes and Coagulation, a Coagulation Factor. In: Biology of Horseshoe Crabs, Sekiguchi, K. (Ed.). Science House Co. Ltd., Tokyo, pp: 334-353.
- Shigenaga, T., Y. Takayenoki, S. Kawasaki, N. Seki and T. Muta *et al.*, 1993. Separation of large and small granules from horseshoe crab (*Tachypleus tridentatus*) hemocytes and characterization of their components. J. Biochem., 114: 307-316.

- Synder, G.K. and C.P. Mangum, 1982. The Relationship Between the Capacity for Oxygen Transport, Size, Shape and Aggregation State of an Extracellular Oxygen Carrier. In: Physiology and Biology of Horseshoe Crab: Studies on Normal and Environmentally Stressed Animals, Bonaventura, J., C. Bonaventura and S. Tesh (Eds.). A.R. Liss Inc., New York, USA., ISBN: 9780845100813, pp 173-188.
- Tincu, J.A. and S.W. Taylor, 2004. Antimicrobial peptides from marine invertebrate. *Antimicrob. Agents Chemother.*, 48: 3645-3654.
- Toh, Y., F. Amizutani, T. Tokunaga and S.I. Muta, 1991. Morphology of the granular hemocytes of the Japanese horseshoe crab *Tachypleus tridentatus* and immunocytochemical localization of clotting factors and antimicrobial substances. *Biomed. Life Sci.*, 266: 137-147.